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Targeted eicosanoids lipidomics of exhaled breath condensate in healthy subjects

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ABSTRACT

Background: Exhaled breath condensate collection is a non-invasive method of sampling the respiratory tract that can be repeated several times in a wide range of clinical settings. Quantitation of non-volatile compounds in the condensate requires highly sensitive analytical methods, e.g. mass spectrometry.
Objective: To validate cross-platform measurements of eicosanoids using high performance liquid chromatography or gas chromatography coupled with mass spectrometry in exhaled breath condensate sampled from 58 healthy individuals.
Methods: Twenty different eicosanoid compounds, representing major arachidonic acid lipoxygenation and cyclooxygenation pathways were measured using a stable isotope dilution method. We applied a free palmitic acid concentration as a surrogate marker for the condensate dilution factor.
Results: Eicosanoids concentrations in the condensates were consistent with their content in other biological fluids. Prostaglandin E_2 was the most abundant mediator, represented by its stable metabolite tetranor-PGEM. Prostaglandin D_2 products were at low concentration, while hydroxyacids derived from lipoxygenation were abundant. 5-HETE was elevated in current tobacco smokers. Leukotriene B_4 has the highest concentration of all 5-LO products. 15-LO analogues of cysteinyl leukotrienes–eoxins were detectable and metabolized to eoxin E_4. Two main vascular prostanoids: prostacyclin and thromboxane B_2 were present as metabolites. A marker for non-enzymatic lipid peroxidation, 8-iso-$PGF_{2\alpha}$ isoprostane was increased in smokers.
Conclusion: Presented targeted lipidomics analysis of exhaled breath condensate in healthy subjects justifies its application to investigation of inflammatory lung diseases. Measurements of non-volatile mediators of inflammation in the condensates might characterize disease-specific pathological mechanisms and responses to treatment.

1. Introduction

Breath, in several cultures, has been a primordial substance associated with origin of life, as well as life's essential manifestation. Yet in medicine, assessment of chemistry of breath has begun only recently. It came about well over 100 years after the atmospheric air was liquefied [1] and has been based on a similar principle of cooling breath into a liquid state. Thus, in commercial or custom-made devices, during tidal breathing, exhaled air is directed through the one-way inspiratory valve to a cooling trap, resulting in accumulation of a clear liquid. About 99% of it consists of water, but the remaining 1% is of utmost interest [2]. This tiny fraction is composed of both volatile and non-volatile compounds that include several biomarkers. Their origin is not clear. It is assumed that they are present in the liquid lining of the airway surface; they become aerolized and carried up during turbulent airflow.

Exhaled breath condensate (EBC) is of great interest to clinicians. Its collection is a completely non-invasive method of sampling of the respiratory tract that can be repeated several times. Collection devices are portable and can be used in a wide range of settings, including outpatient clinics, intensive care units, workplaces, and at home. The method offers a new insight into pathology of respiratory tract and holds promise for clinical utility. Still, unresolved questions remain, especially sensitivity of the assay techniques (ELISA in most studies) for many biomarkers, contributing to the reported variability [3]. This is particularly true for lipids, present in very low concentrations in EBC. Yet, their role as signaling and inflammatory molecules is being recognized.

Mass spectrometry occupies a leading position in the characterization, identification and quantitation of lipids. Its use has led to emergence of lipidomics, defined as the large-scale study of cellular lipids [4–7] (i.e. the lipidome). Mass spectrometry was applied sporadically for measurement of single lipid mediators in EBC [8]. Here we present high performance liquid chromatography–tandem mass spectrometry (HPLC–MS^2) and gas

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chromatography–mass spectrometry (GC–MS) techniques focused on the "targeted" lipidomic analysis of multiple derivatives of arachidonic acid.

2. Methods

2.1. EBC collection

In a pilot study we compared the results of cys-LTs determination in EBC obtained with two instruments: ECO Screen I and II, both products of Jeager (GmbH Hoechberg, Germany). The measurements were substantially lower with the use of the later than the former device. This was due to adsorption of eicosanoids to large surface of plastic bags present in ECO Screen II, but not I, as already noticed by Tufvesson [9]. Therefore, all further experiments were carried out with ECO Screen I. EBC was collected according to ATS/ERS recommendations [2]. Following a tidal breathing for 15 min, 1–2 mL of clear fluid was collected. It was immediately deep frozen before furthermore processing in 1–2 months.

2.2. Subjects studied

Healthy individuals ($n = 58$; 20 males) were recruited among the university hospital staff. Their mean age was 28.9, standard deviation 11.6 years, range 18–72 years, with no age difference between men and women. There were 17 tobacco smokers (11 females and 6 males). The subjects gave an informed consent to participate in the study; the protocol was approved by the University Ethics Committee.

2.3. Sample preparation

Exhaled breath condensates were immediately frozen in −70 °C in 1 mL aliquots and stored until extraction. After thawing on ice the pH of the condensate (1–2 mL) was adjusted to 3.5 using acetic acid (2 μL) and added internal deuterated standards containing: 5-HETE-d_8, 12-HETE-d_8, 15-HETE-d_8; LTD_4-d_5, LTE_4-d_3, LTB_4-d_4 (0.5 ng), tetranor-PGEM-d_6 (5 ng), PGE_2-d_4, PGD_2-d_4, 9α,11β-PGF_2-d_4 (0.25 ng), 6-keto-$PGF_{1\alpha}$-d_4, 8-iso-$PGF_{2\alpha}$-d_4, 11-dehydro-TXB_2-d_4 (0.5 ng, all purchased from Cayman Chemical Co., Ann Arbor, MI, USA), palmitic acid-d_4 (40 ng, Dr Ehrenstorfer GmbH, Augsburg, Germany) in methanol. The sample was extracted twice with 1 mL tert-butylmethyl-ether. Organic phases were combined and evaporated under nitrogen at room temperature. Residue was dissolved in 60 μL of methanol and immediately used for further analyses.

2.3.1. High performance liquid chromatography–tandem mass spectrometry

EBC extract (10 μL, in methanol) was injected on reverse phase column (Zorbax Eclipse XDB C-18, Agilent Technologies, Inc. Santa Clara, CA, USA) stabilized thermally at 37 °C and a gradient consisting of two mobile phases: A acetonitrile/water/acetic acid (20/80/0.0001) and B acetonitrile/iso-propanol/acetic acid (55/45/0.0001, v/v) was used to elute eicosanoids with the flow rate 0.11 mL/min using HPLC equipped with autosampler (Shimadzu Sil-2-AC, Shimadzu Scientific Instruments, Inc. Columbia, MD, USA). The mobile phase binary linear gradient was 1 min 8% B, 9.5 min 8–95% B, 0.5 min 95% B, 0.5 min 95–100% B, 2 min 100% B. Analytes were measured using multiple reaction monitoring mode (MRM) tandem mass spectrometry (Qtrap 4000, Applied Biosystems, Foster City, CA, USA) equipped with electrospray ion source, in three separate runs: positive ionization, negative ionization [10] and the third one time-split into negative ionization for 11-dehydro-TXB2 and tetranor-PGEM, followed by positive one for LTC_4 trans-LTC_4 and LTD_4 to avoid sensitivity loss during multiple ions measurements. Positive ionization was used for: eoxins and LTC_4, trans-LTC_4. Negative ionization was used for prostanoids, LTE_4, LTB_4 and HETEs. Abbreviations and chemical names of the analytes are summarized in Table 1. Ionization mode, molecular and fragmentation ions, retention time, and internal standard used are summarized in the Tables 2 and 3.

2.3.2. Gas chromatography–mass spectrometry

EBC extracts were prepared by a three step derivatization to pentafluorobenzyl ester, trimethylsilyl esters and methoxyoxime, which modified carboxyl, hydroxyl and keto groups of eicosanoids, and purified by a thin-layer chromatography [11]. A gas chromatography negative–ion chemical ionization mass–spectrometry (GC–NICI–MS) was used to measure the prostanoids and palmitic acid (model Engine 5989B series II Helwett Packard, Palo Alto, CA, USA). All the solvents were of HPLC grade and purchased from Mallincrodt Baker, Inc. Phillipsburg, NJ, USA), while other chemicals were from Sigma–Aldrich Co. St. Louis, MO, USA).

Table 1
Measured compounds – abbreviations, chemical names and metabolic pathway.

Abbreviation	Chemical name	Metabolic pathway
5-HETE[a]	5S-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid	5-LO, hydroxyeicosatetraenoic acid
LTB_4	5S,12R-dihydroxy-6Z,8E,10E,14Z-eicosatetraenoic acid	5-LO, leukotriene
LTC_4	5S-hydroxy,6R-(S-glutathionyl),7E,9E,11Z,14Z-eicosatetraenoic acid	5-LO, cysteinyl leukotriene
trans-LTC_4	5S-hydroxy-6R-(S-glutathionyl)-7E,9E,11E14Z-eicosatetraenoic acid	5-LO, cysteinyl leukotriene isomer
LTD_4	5S-hydroxy-6R-(S-cysteinylglycinyl)-7E,9E,11E,14Z-eicosatetraenoic acid	5-LO, cysteinyl leukotriene
LTE_4	5S-hydroxy,6R-(S-cysteinyl),7E,9E,11Z,14Z-eicosatetraenoic acid	5-LO, cysteinyl leukotriene
12-HETE[a]	12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid	12-LO, hydroxyeicosatetraenoic acid
15-HETE[a]	15S-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid	15-LO, hydroxyeicosatetraenoic acid
EXC_4	15S-hydroxy,14R-(S-glutathionyl)-5Z,8Z,10E,12E-eicosatetraenoic acid	15-LO, 14,15-LTC_4, eoxin C_4
EXD_4	15S-hydroxy,14R-(S-cysteinylglycinyl)-5Z,8Z,10E,12E-eicosatetraenoic acid	15-LO, 14,15-LTD_4, eoxin D_4
EXE_4	15S-hydroxy,14R-(S-cysteinyl)-5Z,8Z,10E,12E-eicosatetraenoic acid	15-LO, 14,15-LTE_4, eoxin E_4
LXA_4	5S,6R,15S-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid	LOX, lipoxin, lipoxin A_4
PGE_2	9-oxo-11R,15S-dihydroxy-5Z,13E- prostadienoic acid	COX, prostanoid, prostaglandin E_2
Tetranor-PGEM	11R-hydroxy-9,15-dioxo-2,3,4,5-tetranor-prostan-1,20-dioic acid	COX, prostanoid, prostaglandin E_2 metabolite
PGD_2	9S,15S-dihydroxy-11-oxo-5Z,13E- prostadienoic acid	COX, prostanoid, prostaglandin D_2
9α,11β-PGF_2	5Z,13E,15S,9 alpha,11 beta,15-trihydroxyprosta-5,13-dienoic acid	COX, prostanoid, prostaglandin D_2 metabolite
$PGF_{2\alpha}$	9S,11R,15S-trihydroxy-5Z,13E-prostadienoic acid	COX, prostanoid, prostaglandin $F_{2\alpha}$
6-Keto-$PGF_{1\alpha}$	6-oxo-9S,11R,15S-trihydroxy-13E-prostenoic acid	COX, prostanoid, prostacyclin metabolite
11-Dehydro-TXB_2	9S,15S-dihydroxy-11-oxo-thromboxa-5Z, 13E-dien-1-oic acid	COX, prostanoid, thromboxan B_2 metabolite
8-iso-$PGF_{2\alpha}$	8S,12R,9S,11R,15S-trihydroxy-5Z, 13E-prostadienoic acid	Non-enzymatic, isoprostane F2α
Palmitic acid	Hexadecanoic acid	Saturated fatty acid

[a] Epimers were not distinguished by chromatography; 5-, 12-, 15-LO, LOX – lipoxygenases of arachidonic acid; COX – cyclooxygenases of arachidonic acid.

Table 2
Ionization mode, molecular and fragmentation ions, retention time, and internal standard used in HPLC–MS^2 analysis of EBC.

Analyte – internal standard	Atmospheric pressure electrospray (AP ESI) ionization	Molecular ion $m/z-1$ (negative) $m/z+1$ (positive)	Reaction monitoring ion	Retention time [min]	Internal standard (ion pair)
5-HETE 5-HETE-d_8	Negative	319	257	11.38	327–265
12-HETE 12-HETE-d_8	Negative	319	219	11.16	327–226
15-HETE 15-HETE-d_8	Negative	319	179	10.93	327–184
LXA_4 PGD_2-d_4	Negative	351	217	8.01	355–275
LTB_4 LTB4-d_4	Negative	335	195	9.45	339–197
LTE_4 LTE_4-d_3	Negative	438	333	9.20	441–336
PGE_2 PGE_2-d_4	Negative	351	271	7.29	355–275
PGD_2 PGD_2-d_4	Negative	351	271	7.49	355–275
11-Dehydro-TXB_2 11-Dehydro-TXB2-d_4	Negative	367	161	7.29	371–165
6-Keto-$PGF_{1\alpha}$ 6-keto-$PGF_{1\alpha}$-d_4	Negative	369	163	2.75	373–167
Tetranor-PGEM tetranor-PGEM-d_6	Negative	327	309	1.03	333–315
LTD_4 LTD_4-d_5	Positive	497	189	8.78	502–194
LTC_4 LTD_4-d_5	Positive	626	308	9.03	502–194
trans-LTC_4 LTD_4-d_5	Positive	626	308	9.28	502–194
EXE_4 LTE_4-d_3	Positive	440	205	8.76	443–192
EXD_4 LTD_4-d_5	Positive	497	205	8.24	502–194
EXC_4 LTE_4-d_3	Positive	626	205	8.21	443–192

3. Calculation

Concentration of measured compounds, quantified using a stable isotope dilution method, are presented as medians with the ranges from 25 to 75 centiles. Departure from normal distribution was tested using Shapiro–Wilk statistics. Correlation analyses were performed using Spearman's rank test. Between the groups comparisons were done using non-parametric Mann–Whitney test. Principal component analysis was done with the normally distributed concentrations of eicosanoids and palmitic acid. All calculations were done using STATISTICA (StatSoft, Inc. data analysis software system, version 9.0, www.statsoft.com).

Table 3
Ionization mode, molecular and fragmentation ions, retention time, and internal standard used in GC–MS (negative ions chemical ionization) analysis of EBC.

Analyte – internal standard	Molecular ion $m/z-1$ (negative)	Retention time	Internal standard ion
8-iso-$PGF_{2\alpha}$ 8-iso-$PGF_{2\alpha}$-d_4	569	12.86	573
9α,11β-PGF_2 9α,11β-PGF_2-d_4	569	13.15	573
$PGF_{2\alpha}$ 9α,11β-PGF_2-d_4	569	13.25	573
PGE_2 PGE_2-d_4	524	13.92	528
PGD_2 PGD_2-d_4	524	13.72	528
11-Dehydro-TXB_2 11-dehydro-TXB2-d_4	511	16.75	515
6-Keto-$PGF_{1\alpha}$ 6-keto-$PGF_{1\alpha}$-d_4	614	14.37	618
Palmitic acid Palmitic acid-d_4	255	7.84	259

4. Results

Medians and inter-quartiles ranges of the measured compounds are presented in Table 4.

Statistical analysis of raw data distinguished the metabolites, with normal distribution in EBC samples: PA, LXA_4, 11-dehydro-TXB_2, 6-keto-$PGF_{1\alpha}$, and EXC_4, while the remaining metabolites distribution suggested a greater inter-individual variation. The most abundant eicosanoids in EBC were tetranor-PGEM, 6-keto-$PGF_{1\alpha}$, LTB_4 and 11-dehydro-TXB_2. There was a highly significant positive correlation between PA and tetranor-PGEM ($R=0.77$; $p<0.001$), 6-keto-$PGF_{1\alpha}$ ($R=0.91$; $p<0.001$), LTB_4 ($R=0.88$; $p<0.001$), and 11-dehydro-TXB_2 ($R=0.91$; $p<0.001$). By the principal component analysis of these metabolites and PA, it could be estimated, that PA loaded approximately 30% variance in the covariance matrix of the most abundant EBC eicosanoids. Further comparisons were done assuming PA concentration as a surrogate factor for EBC dilution. Eicosanoids concentrations were recalculated as parts per million (ppm) of PA or picograms per microgram of PA (Table 4).

No gender interaction with measured metabolites was found except 9α,11β-PGF_2, which was significantly higher in men (0.65 [0.35–0.87] vs. 0.35 [0.24–0.55] ppm [25–75 centiles]).

There was, however, a methodological issue which caused lack of reliability in measurements of PGE_2 and PGD_2 using HPLC–MS^2 technique. Due to similar chromatography retention time and possible interference with other eicosanoid ions, and yet unidentified compounds, these prostaglandins were reliably measured only by

Table 4
Medians and inter-quartiles ranges of the EBC compounds content.

Compound	Raw concentration per 1 mL median [25–75%] quartiles	Concentration in parts per million of palmitic acid median quartiles [25–75%]
5-HETE (HPLC–MS^2)	2.81 [1.89–5.19]	5.73 [3.12–8.09]
12-HETE (HPLC–MS^2)	3.23 [2.21–4.6]	4.98 [3.54–8.87]
15-HETE (HPLC–MS^2)	3.25 [2.10–4.61]	6.62 [3.55–10.99]
LXA_4 (HPLC–MS^2)	0.22 [0.13–0.30]	0.39 [0.25–0.54]
LTB_4 (HPLC–MS^2)	6.54 [3.82–9.87]	12.53 [9.27–16.84]
LTE_4 (HPLC–MS^2)	1.16 [0.77–1.75]	2.25 [1.38–3.34]
[a]PGE_2 (HPLC–MS^2)	0.58 [0.38–0.82]	0.98 [0.67–1.67]
PGE_2 (GC–MS)	0.93 [0.57–1.60]	1.77 [1.02–3.24]
[a]PGD_2 (HPLC–MS^2)	0.52 [0.40–0.73]	0.96 [0.73–1.51]
PDG_2 (GC–MS)	0.42 [0.29–0.96]	0.90 [0.51–1.53]
11-Dehydro-TXB_2 (HPLC–MS^2)	4.24 [3.76–4.82]	8.10 [5.77–10.22]
11-Dehydro-TXB_2 (GC–MS)	4.25 [3.69–4.80]	7.70 [6.02–10.00]
6-Keto-$PGF_{1\alpha}$ (HPLC–MS^2)	11.60 [8.55–14.40]	19.17 [15.83–25.06]
6-Keto-$PGF_{1\alpha}$ (GC–MS)	11.41 [7.94–14.90]	20.87 [16.17–24.93]
8-iso-$PGF_{2\alpha}$ (GC–MS)	0.19 [0.14–0.29]	0.43 [0.24–0.62]
9α,11β-PGF_2 (GC–MS)	0.24 [0.16–0.44]	0.39 [0.28–0.69]
$PGF_{2\alpha}$ (GC–MS)	0.28 [0.14–0.47]	0.47 [0.27–0.70]
Tetranor-PGEM (HPLC–MS^2)	158.3 [115.5–250.4]	263.7 [197.4–428.9]
LTD_4 (HPLC–MS^2)	1.98 [1.05–2.80]	3.87 [1.89–5.46]
LTC_4 (HPLC–MS^2)	1.91 [0.89–3.37]	2.92 [1.40–5.46]
trans-LTC_4 (HPLC–MS^2)	6.37 [1.82–10.72]	9.54 [2.84–18.62]
EXE_4 (HPLC–MS^2)	3.70 [2.05–10.28]	7.49 [4.01–22.37]
EXD_4 (HPLC–MS^2)	0.73 [0.60–1.53]	1.52 [1.23–2.37]
EXC_4 (HPLC–MS^2)	0.81 [0.55–1.32]	1.86 [0.96–2.91]
Palmitic acid (GC–MS)	536.9 [411.4–655.6]	–

[a] Poor accuracy of HPLC–MS^2 measurements of PGE_2 and PGD_2. Eicosanoids in pg/mL, palmitic acid in ng/mL.

GC–MS technique. The most abundant cyclooxygenase metabolite in EBC, tetranor-PGEM was present at hundred-fold higher levels than PGE_2. For similar reason, tetranor-PGDM could be the main PGD_2 metabolite, instead of 9α,11β-PGF_2 the level of which was roughly a half of the PGD_2 concentration.

Table 5
Comparison of EBC eicosanoids between non-smoking subjects and current smokers.

Analyte	Non-smoking subjects	Current smokers	*p*
5-HETE	4.29 [2.94–7.55]	8.06 [5.81–14.35]	0.008
12-HETE	4.88 [3.53–8.03][	6.16 [3.81–8.84]	n.s.
15-HETE	5.34 [3.48–11.13]	7.46 [3.86–10.96]	n.s.
LXA_4	0.39 [0.25–0.53]	0.35 [0.22–0.67]	n.s.
LTB_4	12.0 [10.19–16.35]	15.16 [8.96–19.03]	n.s.
LTE_4	1.81 [1.31–3.45]	2.47 [1.64–3.80]	n.s.
PGE_2	0.91 [0.69–1.63]	1.22 [0.66–1.68]	n.s.
PGD_2	0.96 [0.76–1.55]	1.05 [0.77–1.59]	n.s.
11-Dehydro-TXB_2	7.81 [5.49–9.50]	8.64 [7.26–10.18]	n.s.
6-Keto-$PGF_{1\alpha}$	19.77 [15.82–24.38]	18.79 [14.87–30.11]	n.s.
Tetranor-PGEM	266.2 [191.0–442.5]	247.85 [197.4–415.0]	n.s.
LTD_4	3.52 [1.86–4.72]	4.51 [3.17–7.04]	0.06
LTC_4	2.59 [1.34–5.57]	4.35 [1.59–7.71]	n.s.
trans-LTC_4	11.34 [2.16–17.57]	8.38 [2.90–24.02]	n.s.
EXE_4	5.46 [2.64–21.1]	9.37 [6.31–27.14]	n.s.
EXD_4	1.35 [0.97–2.01]	2.12 [1.39–5.95]	0.07
EXC_4	1.44 [0.67–2.53]	2.94 [0.95–3.18]	n.s.
GC–MS analyte			
8-iso-$PGF_{2\alpha}$	0.37 [0.20–0.54]	0.60 [0.39–0.79]	0.016
9α,11β-PGF_2	0.37 [0.25–0.66]	0.36 [0.33–0.79]	n.s.
$PGF_{2\alpha}$	0.52 [0.23–0.70]	0.39 [0.28–0.70]	n.s.
PGE_2	1.57 [1.06–2.81]	2.66 [1.07–5.18]	n.s.
PGD_2	0.96 [0.56–1.50]	0.73 [0.48–1.59]	n.s.
11-Dehydro-TXB_2	7.58 [5.33–9.01]	8.49 [6.94–10.17]	n.s.
6-Keto-$PGF_{1\alpha}$	20.98 [16.4–23.58]	20.77 [14.27–28.30]	n.s.
Palmitic acid (PA)	600.3 [436.7–677.6]	457.9 [417.0–536.5]	n.s.

Palmitic acid concentration in ng/mL of EBC, all other concentrations are ppm of palmitic acid. Median and 25–75 centiles range is given. *p*-Type I error of Mann–Whitney *U*-test.

In the group of lipoxygenation products a significant rank correlation was observed between 5-HETE and LTB_4 ($R = 0.35$; $p < 0.01$) but not with any cysteinyl leukotrienes. Cysteinyl leukotrienes were intercorrelated: LTC_4 and trans-LTC_4 ($R = 0.78$; $p < 0.001$), LTD_4 ($R = 0.58$; $p < 0.001$), LTE_4 ($R = 0.43$; $p < 0.001$). It was intriguing, that LTC_4 was negatively correlated with LXA_4 ($R = -0.30$; $p = 0.026$), because these compounds have opposite effects on inflammation. 15-HETE concentrations did not correlate with eoxins, but EXC_4 was positively correlated with EXD_4 ($R = 0.48$; $p < 0.01$) and negatively correlated with LXA_4 ($R = -0.39$; $p = 0.03$). 12-HETE correlated positively with LXA_4 ($R = 0.31$; $p = 0.02$) but not with thromboxane or prostacyclin metabolite, suggesting its extravascular origin.

4.1. Tobacco smoking and eicosanoids in EBC

Comparison of non-smoking subjects with the current tobacco smokers revealed some striking differences in eicosanoid content of EBC (Table 5). Current smokers had higher concentrations of 5-HETE (8.06 [5.81–14.35] vs. (4.29 [3.01–7.43] ppm [25–75 centiles]; $p < 0.01$) and 8-iso-$PGF_{2\alpha}$ (0.60 [0.38–0.79] vs. 0.37 [0.19–0.55]; $p = 0.02$). A statistical trend was present in contrasts for LTD_4 (4.51 [3.17–7.04] vs. 3.52 [1.82–4.73]; $p = 0.06$) and EXD_4 (2.12 [1.39–5.95] vs. 1.21 [0.99–1.97]; $p = 0.06$), but not for LTB_4 ($p = 0.70$). Number of cigarettes smoked daily correlated positively with 5-HETE ($R = 0.42$; $p = 0.003$) and with 8-iso-$PGF_{2\alpha}$ ($R = 0.33$; $p = 0.02$).

5. Discussion

The aim of the current study was to develop an analytical approach based on mass spectrometry measurements of eicosanoids of healthy subjects in EBC. Due to the extremely low concentrations in EBC, close to detection threshold of immunoenzymatic assays (~7.5 pg/mL), and a small sample volume, quantitative analysis of eicosanoids is very limited [9]. Fast non-invasive method of collection and ability to repeat sampling gives EBC an advantage over induced sputum or bronchoalveolar lavage. Eicosanoids comprise hundreds of bioactive compounds, derivatives of arachidonic acid. They are pro-inflammatory, or less frequently anti-inflammatory mediators. Except for isoprostanes, biosynthesis of eicosanoids is strictly regulated by modulation of phospholipases activity and expression of cyclooxygenases, lipoxygenases and synthases. All eicosanoids undergo rapid metabolism, which is critical for their pharmacological effects [10].

We complemented this focused lipidomics using two different analytical platforms and ascertained eicosanoids levels, there possible, in parallel. Reverse phase high performance liquid chromatography offers separation of chemical compounds based on their hydrophobic interactions and molecular diameter and can be fine tuned by change of elution gradient and the column properties. It is conveniently coupled to atmospheric pressure electrospray ionization and tandem mass spectrometry. In this technique compounds sorted on their chromatography retention time are further separated by difference of source and fragmentation ions mass to charge ratio [11]. With gas chromatography, chemical modification of similar molecules by substitution of their carboxyl or hydroxyl groups, enables their retention time differences to be amplified. In this method a single quadrupole instrument is usually sufficiently sensitive, because ionization is highly effective in chemical ionization mode with the help of additional methane molecules [12]. In the current study internal deuterated standards were used to ensure compensation for EBC extraction or chromatography injection errors. If no compound with identical chemical formula was available in the deuterated form, the closest one was selected. This

stable isotope dilution method is a standard approach in quantitative mass spectrometry [6,11,12].

However, EBC collection is biased by a variable dilution of the sample with condensed water vapor. A few methods to adjust the dilution factor of a sample, similarly to recalculation of urinary metabolites per creatinine content, have been proposed [13–16]. We used palmitic acid concentration, as a marker for dilution of EBC sample. Palmitic acid is the most abundant non-volatile lipid molecule in EBC, because phospholipid esters of palmitic acid comprise over 60% of the lung surfactant. Yet, using our analytical procedure these phospholipids were excluded from measurement because much more aggressive chemical conditions would be required to hydrolise esterified palmitic acid [17]. The mean concentration of free palmitic acid in EBC was 560 ng/mL and its standard deviation was moderate (187 ng/mL). It did not depend on gender, age or tobacco smoking. Unesterified palmitic acid blood plasma concentration is in the submillimolar range (81–86 μmol/L) [18] while the average EBC level we found, has been roughly 40 times lower (2.31 μmol/L). This is consistent with passive diffusion of albumin-adsorbed free fatty acid from the vascular bed into airways compartment of the lung. To estimate dilution factor of non-volatile constituents of EBC a current conducting ions [13], total protein [14], tyrosine [15] or urea [16] were proposed as other diffusible biomolecules. However, measurements of micromolar concentrations of these compounds cannot be done using routine clinical laboratory analyzers and require development of stand-alone methods. Moreover, to estimate dilution of EBC sample by measurement of electric conductance, volatile ions have to be removed by evaporation prior to the measurement and an extra sample volume is needed. In our opinion, recalculation of eicosanoids content on palmitic acid concentration in EBC has the advantage of simultaneous processing of the compounds and unbiased measurement using GC–MS. It is probably a surrogate marker only for dilution of EBC with water, however recalculated results can improve statistics. For example, difference in 5-HETE concentration between non-smokers and smokers, significant also for raw data, was greater following recalculation on PA (chi square approximation changed from 4.81 to 6.95).

Ascertainment of several eicosanoids in EBC, interlinked by common metabolic pathways, gave results consistent with their estimations in other biological fluids. Prostaglandin E_2 is the most abundant eicosanoid mediator in the lung, however, only a tiny fraction (0.67%) is not metabolized, while the rest is represented by tetranor-PGEM, the stable metabolite. Prostaglandin D_2 production in the lung of healthy subjects is small, as also judged from its inactive 9α,11β-PGF_2 metabolite concentration. Enzymatic lipoxygenation products are quite abundant in EBC of healthy subjects and their concentrations are all within similar range. Gender dimorphism of eicosanoids biosynthesis was previously studied using either sex hormones stimulation of cells in vitro, or by a comparison of urinary excretion of metabolites in clinical studies. Basal urinary outputs of prostacyclin and thromboxane metabolites are similar for healthy women and men [18]. Differences following activated biosynthesis during physical exercise or inflammatory diseases generally reflected increased capacity for cyclooxygenation of arachidonic acid and its release by phospholipases, which was higher in men. Urinary leukotriene E_4 excretion is higher in women for unknown reason [19]. 5-HETE increases in response to tobacco smoking, although no increase in leukotrienes has been noted in contrast to the finding by Carpagnano et al. [20] done using immunoassay, probably due to insufficient statistical power of our study and less intense smoking (80% subjects smoked < 1 pack day^{-1}). Leukotriene B_4 has the highest concentration of all 5-lipoxygenase (5-LO) products; biologically inactive trans-LTC_4 is also abundant. All three cysteinyl leukotrienes have similar concentrations, but the pattern of their 15-LO analogues–eoxins was quite different. It seems, that in healthy subjects eoxins C_4 and D_4 are rapidly metabolized to eoxin E_4. Two main vascular prostanoids, thromboxane B_2 and prostacyclin are present in EBC at relatively high concentrations. Lack of correlation between prostaglandins and thromboxane or prostacyclin suggests their production in different lung tissue compartments. Lipoxin A_4, the only anti-inflammatory eicosanoid measured in the current study, has very low concentration in EBC of healthy subjects, which is in the similar range as isoprostane 8-iso-$PGF_{2\alpha}$. The latter was also increased in smokers as observed by Hoffmeyer et al. [21] using immunoassay.

Presented targeted lipidomics analysis of exhaled breath condensate in healthy subjects points to feasibility of similar investigation in lung diseases. Using a non-invasive sampling of exhaled breath condensate and chromatography–mass spectrometry, non-volatile mediators of inflammation could be measured and characterize disease-specific pathological mechanisms as well as responses to treatment.

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